IJBST

ISSN: 0974 – 3987
IJBST (2009), 2(6): 68-75

Biodegradation of pyridine containing wastewater in a batch aerobic biological reactor utilizing an enriched bacterial culture

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ABSTRACT

The degradation of pyridine containing wastewater was studied in a batch aerobic biological reactor using enriched bacterial culture. The culture was developed taking soil from the sewage line of a pilot plant used for the manufacture of N,N'-dichloro bis(2,4,6-trichlorophenyl)urea (CC2) as innoculum. The bacteria was isolated and found to be *Bacillus* species. It was effective in degradation of pyridine containing wastewater. The effects of parameters namely, airflow rate and initial concentration on the biodegradation were studied. 82% degradation of pyridine was achieved within 26 hrs and 88% degradation of pyridine was achieved with in 45 hrs in the slurry bio reactor. A maximum of 83% chemical oxygen demand removal was achieved during the degradation process. The Yield coefficient of suspended biomass was determined.

Key words: Biodegradation, CC2, kinetics, pyridine, wastewater, bioreactor

INTRODUCTION

Pyridine is discharged into the atmosphere through effluents from a wide variety of industries [1-5]. It is also discharged along with the effluent from manufacturing plant of N,N'-dichlorobis(2,4,6-trichlorophenyl)urea (CC2), a chemical decontaminant of sulphur mustard, a known chemical warfare agent [6]. The manufacturing plant of CC2 produces wastewater containing pyridine, acetic acid, diphenyl urea (DPU) [6-8]. Pyridine is a toxic aromatic nitro-compound with obnoxious odour [9]. Pyridine and its derivatives have also shown mutagenic potency [10]. Due to its moderate to acute toxicity the USEPA has listed pyridine as one of the priority organic pollutants [11].

Hence the pyridine present in the wastewater must be treated before releasing into the environment. There are a number of physico-chemical methods for treatment of wastewater containing pyridine namely, adsorption, incineration and aqueous oxidation [12]. But these processes are not preferred since they are highly cost and energy intensive. On the other hand biological treatment processes employing microorganisms for the treatment of wastewater are preferred due to low cost and their environmentally-benign nature. In recent years most of the hazardous organic compounds released in the environment are biologically treated due its eco-friendly nature [13-15]. Hence, for treatment of wastewater containing pyridine biological processes are preferred.

A number of reports are available for the degradation of pyridine in flask culture experiments using pure cultures of *Bacillus coagulants*, *Micrococcus luteus*, *Nocardiodes* sp. and *Pseudomonas* sp. [9, 16-19]. A few reports are available on biodegradation of pyridine using a bioreactor. A detailed investigation on pyridine biodegradation in a CMAS reactor, using an isolated bacterial culture of *Pseudomonas pseudoalcaligenes* – KPN, as a starter inoculum was reported recently (Padoley et al. 2006). The results of this study indicated that pyridine could be degraded efficiently at an optimal hydraulic retention time (HRT) of 24 h. Pyridine was used as the sole source of carbon and nitrogen by the biomass. Ammonia-nitrogen (NH_3-N) was formed due to the metabolism of the pyridine ring. Very few reports are available on biodegradation of pyridine in presence of other organic compounds [20]. In their study pyridine was used as only source of nitrogen.

This study addresses a detailed investigation on biodegradation of wastewater containing pyridine, acetic acid and DPU. The objective of the present study was to evaluate efficacy of biological process to degrade pyridine and pyridine containing wastewater discharged from a pilot plant producing CC2 [8].

So far no reports are available, in which pyridine is degraded in presence of another source of nitrogen. The effect of parameters namely, airflow rate, initial concentration of pyridine and initial concentration of biomass in the slurry bioreactor on biological degradation of the wastewater were also investigated.

MATERIALS AND METHODS

Experimental setup

A three phase gas-liquid-solid batch aerobic biological reactor was used for biodegradation study in the present work (Figure 1). The bioreactor was constructed from glass and had an internal diameter of 130 mm and a working volume of 2 L. The bioreactor was fitted with a double coil condenser for condensing the evolved vapour from the reactor. A four flat-blade disk turbine impeller was fixed in the reactor for agitation. Air entered at the base through a sintered glass distribution ball. The airflow rate (Q) was measured by a calibrated rotameter. The bioreactor was placed on a heating mantle, which was connected with a temperature indicator controller (TIC). One temperature sensor (RTD) and dissolved oxygen (DO) sensor were inserted into the reactor.

Enrichment and identification of bacterial culture for degradation of waste

The soil samples of the site, where the liquid waste of the production plant of CC2 is discharged, were collected. The enrichment of biodegrading bacteria for pyridine was carried out in the basal medium, which contains 45 mg L^{-1} pyridine. The enrichment was also carried out with mixture of pyridine (45 mg L^{-1}) and acetic acid (60 mg L^{-1}) and actual waste separately. One percent by weight of soil samples were added to 300 ml medium 1.0 L flask for the enrichment and incubated at 37 ^{0}C for 7 days (200 rpm). The basal medium contains yeast extract 100 mg L^{-1}, K_2HPO_4 43.0 mg L^{-1}, KH_2PO_4 17.0 mg L^{-1}, N_2HPO_4 89.2 mg L^{-1}, NH_4Cl 50.0 mg L^{-1}, $MgCl_2$ 45.0 mg L^{-1}, $CaCl_2$12.0 mg L^{-1}, $FeCl_2$ 5.0 mg L^{-1} and $(NH_4)_2SO_4$ 45.0 mg L^{-1}.

Three enrichment cycles were performed using the same experimental conditions except the addition of soil was replaced with the enriched cultures of previous cycle. The pyridine degradation was monitored during all the enrichment cycles. After enrichment, plating was carried out on 1.75% agar-agar containing actual waste (without basal medium) and incubated at 37 ^{0}C for two days. Bacterial colonies were picked up in the broth medium and bacteria were subjected to Gram staining and biochemical tests for identification. The isolated bacteria were grown in a shake flask in the basal medium containing actual waste and pyridine concentration was monitored. The bacterial pellet obtained from the 48 hrs grown shake flask grown culture was used as inoculum for the slurry bioreactor.

Bacterial genomic DNA was isolated using genomic DNA isolation kit (M/s Promega, USA), and the small subunit rRNA gene was amplified using the two universal primers 16S1 (5-GAGTTTGATCCTGGCTCA-3) and 16S2 (5-CGGCTACCTTGTTACGACTT-3). The amplified products were sequenced using automated DNA sequencer (3130, ABI, USA). The deduced sequence was subjected to BLAST search for closest match in the database.

Operation of the batch aerobic biological reactor

Wastewater (Table 1) obtained from the pilot plant that is used for the production of CC2, was treated with sodium hydroxide solution to get a pH of 7.0. It has been reported that maximum pyridine degradation and growth of pyridine degrading organisms occurred in the pH range of 6.8– 7.4 [21]. Hence in our experiment we have used neutralized waste. A volume of 0.95 L of this neutral solution and 50 ml of the basal medium were added into the bioreactor to get the desired final concentration of different nutrient as used in the culture development. The slurry was stirred at an rpm of 180, which was sufficient to keep proper mixing of the slurry in the reactor. The slurry was heated by the heating mantle and the temperature was maintained at 37±1 ^{0}C using the TIC. Air was passed through the slurry from a cylinder and the flow rate was monitored and controlled through a rotameter. A gas-manifold with two air cylinders was used to keep the flow of air constant. Chilled water at a temperature of 2 ^{0}C was passed through the condenser of the reactor to avoid loss of volatile organics by evaporation. Reports are available that a temperature range of 30–37 ^{0}C is optimal for the growth of *Pseudomonas* species isolated from sewage [22, 23]. Hence in our experiments we have used a temperature of 37 ^{0}C. When the temperature of the slurry reached 37 ^{0}C, previously enriched biomass was added into the bioreactor as inoculum and stirring was continued. This time was taken as zero. Samples were taken out from the reactor at different time interval and were analyzed. Each time the three samples of 1 ml each were drawn to get average values by analysis. One controlled experiment was carried out without the addition of biomass to see loss of organic matter by evaporation or adsorption.

Bacterial culture was grown in shake flask and centrifuged at 3700 xg for 5 minutes to obtain the bacteria in pellet and this bio mass was used as inoculum in the bioreactor. From the pellet 1.2 and 1.8 g were used to have an X_0 of 1200 mg L^{-1} and 1800 mg L^{-1} respectively. The MLSS consisted mostly of microorganisms, non-biodegradable suspended organic matter and other inert suspended matter. Since the biomass concentrations changed slowly with time in this work, it was assumed to be time-independent during short time intervals.

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ISSN: 0974 – 3987
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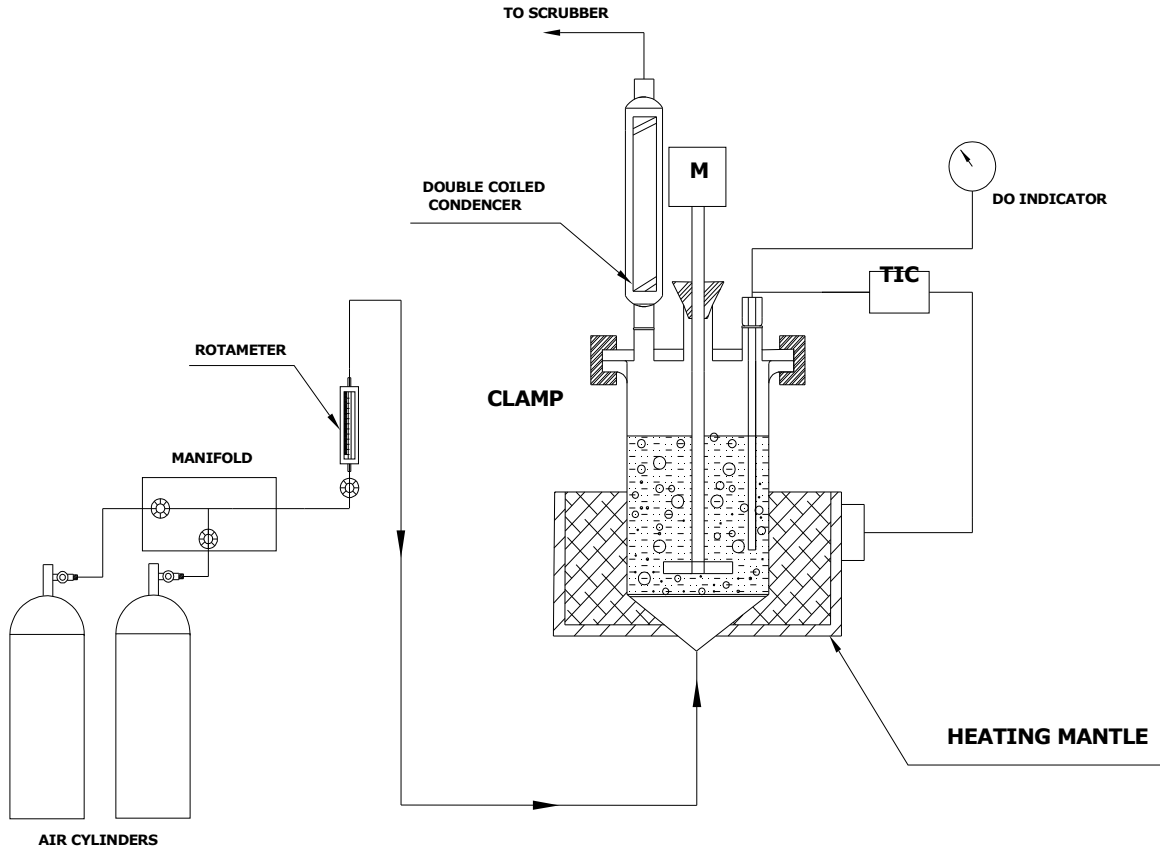

Figure 1. A schematic diagram of the gas–liquid–solid three-phase batch aerobic biological reactor used for biodegradation experiments

Analysis

Samples of the wastewater were taken, centrifuged (SIGMA1-15 K, Germany) at 10000 xg for 5 minutes, decanted and analyzed for concentration of pyridine, acetic acid and DPU using high performance liquid chromatography (HPLC), (Shimadzu, Japan, model LC- 6A) fitted with C-18 column. A UV detector (SPD-6AV) at a wavelength of 257 nm was used for scanning.

The column temperature was kept at 40 ^{0}C. The mobile phase was a mixture of methanol and water (70:30) and the flow rate of the mobile phase was 1.5 ml/min. A volume of 10 µL of each samples was injected into the column through Reheodyne injector. Chromatopac (C-R3A) was used as integrator and recorder.

The mixed liquor suspended solids (MLSS) was used to characterize the biological mass in the waste activated sludge [24]. The bacterial pellet obtained after centrifugation (10000 xg) of samples drawn from the reactor was weighed and used as biomass. Samples drawn from the bioreactor were centrifuged at 10000 xg, and supernatant was used for the measurement of Chemical Oxygen Demand (COD) using American Public Health Association (APHA) standard methods, 1998 under section 5220 [25]. Dissolved oxygen (DO) concentration was monitored using an oxygen meter and electrode (OXI-191, Toshniwal, India).

Table 1. Characteristics of the Wastewater obtained from the Pilot Plant used for the production of CC2

Colour	Light yellow Brownish
pH	3.7-3.9
Total solid	1500-1800 mg L^{-1}
TDS	1250 mg L^{-1}
COD	7000-8000 mg L^{-1}
Conductivity	1100-1200 micro S cm^{-1}
BOD	9800-11200 mg L^{-1}
Pyridine	45-65 mg L^{-1}
Acetic acid	60-80 mg L^{-1}
DPU	10-15 mg L^{-1}

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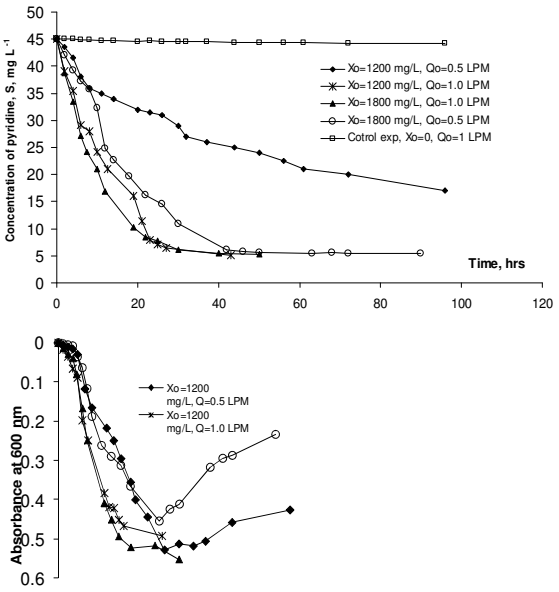

Figure 2. Change of concentration of pyridine and growth characteristics of biomass during biodegradation in the bioreactor at 37 ^{o}C with S_o = 45 mg L^{-1} and with X_o of 1800 mg L^{-1} and 1200 mg L^{-1}

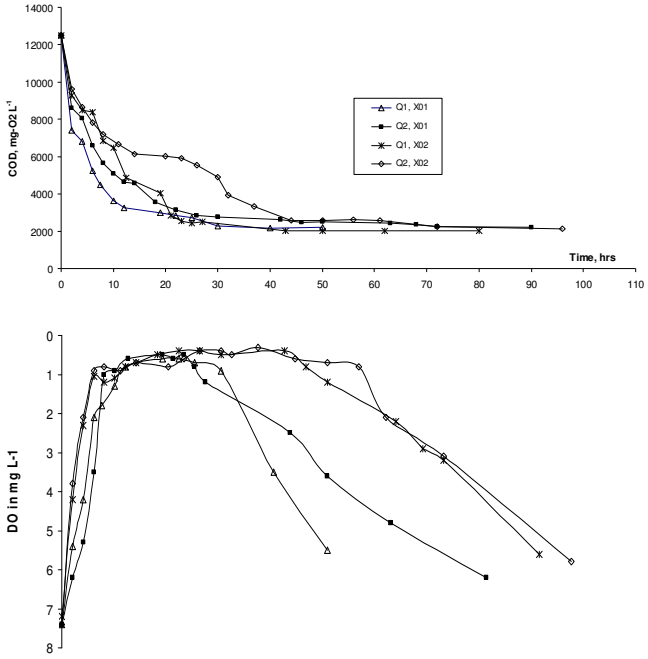

Figure 3. Change of COD and dissolved oxygen concentration during biodegradation of wastewater at 37 ^{o}C having initial COD of 12500 mg L^{-1} in bioreactor where, X_{01}=1800 mg L^{-1}, X_{02}=1200 mg L^{-1}, Q1=1.0 LPM, Q2=0.5 LPM, So=45 mg L^{-1}.

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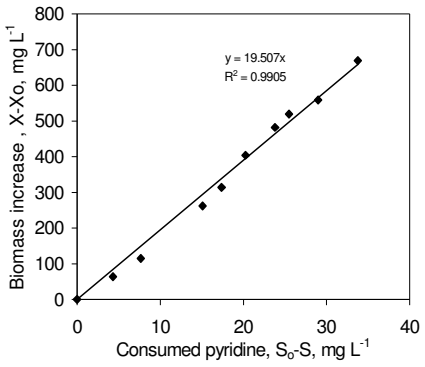

Figure 4. Yield coefficient of suspended biomass in the bioreactor operated at 37 ^{o}C with X_0=1800 mg L^{-1}, Q=0.5 LPM

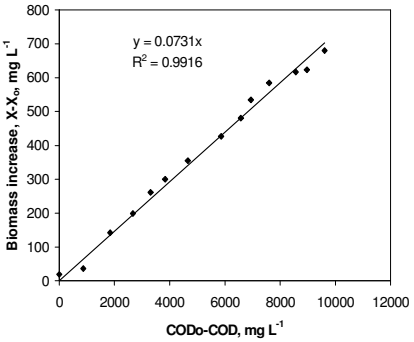

Figure 5. Yield coefficient of suspended biomass in the bioreactor operated at 37 ^{o}C with X_0=1200 mg L^{-1}, Q=0.5 LPM

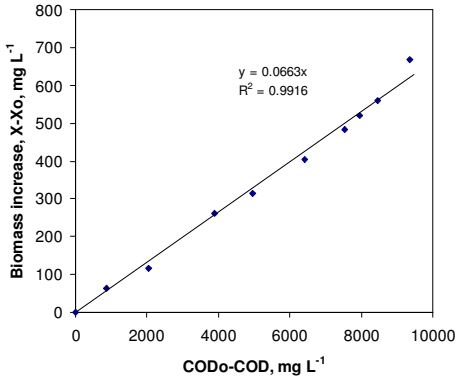

Figure 6. Yield coefficient of suspended biomass in the bioreactor at 37 ^{o}C, X_0=1800 mg L^{-1}, Q=0.5 LPM

RESULTS AND DISCUSSION

Enrichment, isolation and identification

The enrichment from soil samples resulted the in the reduction of pyridine concentration from 45 mg L^{-1} to 13 mg L^{-1} after three cycles which was maximum in actual waste with minimal basal medium. Actual waste enriched culture was plated and allowed to grow on agar medium without basal medium produced colonies with similar morphology i.e., white, smooth, circular, convex colonies. Six representative bacterial colonies were picked up and were inoculated in broth (minimum basal medium) containing actual waste for biodegradation. All bacterial isolates were found to degrade waste at 37 ^{0}C up to 67 % with respect to pyridine.

All bacterial isolated were Gram positive, medium size rods with rounded ends. The 16s rRNA gene sequence was found to be identical thus considered to be of clones of the same isolates and one of these isolates was selected for biochemical characterization and further studies as an example, degradation of waste in slurry bioreactor.

The strictly aerobic *Bacillus* isolate could ferment glucose and was catalase and oxidase positive (Table 2). It exhibited optimum growth at 37 ^{0}C and pH 7. The phylogenetic analysis based on 16s rRNA gene sequence indicated that the bacterial isolate is closely related to the genus *Bacillus*. The closest relative appears to be *Bacillus* sp. 253-3.2-LH-H15(s)-05 and *Bacillus sphaericus* with a similarity of 100%.

Biodegradation

Samples were drawn from the bioreactor at different time and were analyzed by HPLC. It was observed that the concentration of pyridine, acetic acid and DPU were decreasing.

The same observations were made during the bacterial culture development. It was observed that degradation of acetic acid from the mixture was much faster and it degraded almost completely with in few hours of operation of the bioreactor. In this study the decrease in COD of the mixture is basically attributed to the degradation of all the biodegradable organic pollutants present in the wastewater. However the concentration of pyridine was of special interest and was monitored through out the degradation study. Negligible decrease in the concentration of pyridine with time was observed in the control experiments that were carried out without addition of biomass into the reactor (Figure 2). This confirms that the pyridine has neither been lost by evaporation or by sorption.

Therefore in our experiments the decrease in concentration of pyridine is attributed to biological degradation. Degradation of pyridine from a synthetic wastewater containing only pyridine has been reported earlier [26], where pyridine was used by the bacterial mass as only carbon source. In the present study it was observed that pyridine along with acetic acid and DPU was used as the carbon source by the bacteria.

Table 2. Morphological and biochemical characteristics of the Bacillus species

Colony morphology	
Form	Circular
Elevation	Convex
Margin	Smooth
Color	white
Cell morphology	
Shape	Medium rods
Approximate size in diameter	3–5 µm
Motility	Motile
Arrangements	Single
Gram stain	+
Growth temperature	
Optimum	37 ◦C
Biochemical characteristics	
Catalase	+
Oxidase	+
Glucose metabolism	
Oxidative	+
Fermentative	+
Nitrate reduction	
$NO_3 \rightarrow NO_2 - NO_2 \rightarrow N_2$	–
Hydrolysis of	
Gelatin	+
Esculin	+
Anaerobic growth	–
Utilization as a sole carbon source	
Tryptophan	+
Arginine – Urea – Glucose	+
Arabinose	+
Mannose	+
Mannitol	+
N-Acetyl glucosamine	+
Maltose	+

Biodegradation potential of Bacillus species

In the present investigation, the morphological, physiological and biochemical characteristics were evaluated for the potential culture for biodegradation of pyridine from wastewater containing a mixture of compounds. As discussed in the preceding section, *Pseudomonas putida QP2*, *Pseudomonas ayucida IGTN9m* and *Pseudomonas aeruginosa QP* have been reported for biodegradation of pyridine and its derivatives. The cultures of *Pseudomonas sp*. cited in the literature could degrade pyridine and its derivatives in the concentration range of 100–400 mg L^{-1}. In the present study the isolated *Bacillus species* could degrade pyridine efficiently in the concentration range of 10-100 mg L^{-1} from a mixture of compounds. Typical growth characteristics of the biomass in the reactor were obtained (Figure 2). It was observed that with change in X_0 and Q there was no change in the nature of the growth of bacteria and in every situation a growth phase and a lag phase exists.

Effect of airflow rate and initial biomass concentration on degradation

The reactor was operated with different Q and initial MLSS (X_0) in order to evaluate the effect of these parameters on biodegradation. The degradation of pyridine from wastewater containing a mixture of compounds was found to be efficient (Figure 2). It was observed that the concentration of pyridine was reduced by 88% degradation of pyridine is achieved within 45 hrs when the airflow rate, Q is 1.0 LPM with X_0 of 1800. Whereas with Q of 0.5 LPM, only 39% degradation of pyridine is achieved within 45 hrs with X_0 of 1200mg L^{-1}. In all the cases at the end of experiments a residual pyridine (10-12%) was observed due to the limitation of biomass. The nutrients, i.e., vitamins, minerals etc., other than the carbon sources may be responsible for the decline in bacterial growth.

In the degradation process pyridine is getting degraded along with the other biodegradable organics present in the wastewater by the biomass. The COD of the wastewater in the reactor was determined to evaluate the degradation of the mixture of organic pollutants. Almost 75 % COD removal was achieved in all operating conditions except only when X_0 of 1200 mg L^{-1} with Q of 0.5 LPM (Figure 3). Almost in all cases maximum of 82% COD removal is achieved. From this study it is clear that 88% of pyridine degradation is possible from the wastewater where as 83% COD removal is possible by biodegradation.

Oxygen consumption

The change in DO during the process of biodegradation in the reactor was monitored. The DO level with X_0=1200 mg L^{-1} and Q=1.0 sharply decreased 1.2 mg L^{-1} in 8 hrs and further reduced to 0.6 mg L^{-1} in 19 hrs (Figure 3) and then the DO started sharply increasing and reached the saturated value. Almost similar observations were made in other situations. The change in DO is related to the degradation of the organic pollutants present in the wastewater. The concentration of pyridine at Q=1.0 LPM decreased sharply up to 22.0 hrs to 8.5 mg L^{-1} and during this time the DO has also decreased sharply to 0.6 mg L^{-1} and then started increasing. Figure 3 illustrates the relationship between the change in the dissolved oxygen concentration and the decrease in COD during the process of biodegradation in reactor. Sharp decrease in COD is observed up to 12 hours for both Q=0.5 and 1.0 LPM and then rate of decrease in COD is reduced. The increase in DO of the mixture was observed after 22 hrs of operation of the reactor. At Q of 0.5 LPM, the DO was limiting for a longer time which resulted slower biological degradation.

Biokinetic parameters

The data of suspended biomass (Figure 2) represent a typical growth and decay curve with a well defined growth phase, followed by a constant growth phase, and the death phase. The concentration data of pyridine and suspended biomass facilitate a priori estimation of biokinetic parameters for evaluating the growth rate of suspended biomass and pyridine utilization. The yield coefficient (Y) for suspended biomass is assumed approximately constant over the range of pyridine concentrations encountered in the growth phase. The yield coefficient for pyridine-utilizing bacteria was determined from the slope of a linearized plot for the increase in the concentration of suspended biomass ($X-X_0$) versus decrease in pyridine concentration (S_0-S), which was 19.507 mg MLSS mg^{-1} pyridine (Figure 4). This value is very high and is not giving the actual coefficient because the bacteria used pyridine and other organic pollutants from the wastewater for growth. COD removal is directly related to the degradation of the organic pollutants that are present in the wastewater. The yield coefficient is 0.0731 when COD removal is considered for the mixture of pollutants present in the wastewater with X_0=1200 mg L^{-1}, Q=0.5 LPM and COD_0=12500 mg L^{-1} (Figure 5). Similarly, when the data of experiments with X_0=1800 mg L^{-1}, Q=0.5 LPM and COD_0=12500 mg L^{-1} is analyzed the value of Y was 0.0663 (Figure 6). The insignificant variation between the values of Y obtained at X_0=1200 mg L^{-1} and X_0=1800 mg L^{-1} validates our experimental data.

The data in the death phase can be used for estimating the decay coefficient of suspended biomass [27]. The decay coefficient, b, was determined from the slope of a plot of $-\ln(X_2/X_1)$ vs (t_2-t_2), where t_1 and t_2 are the initial and final time; X_1 and X_2 are the initial and final biomass concentration, respectively. The value of decay coefficient was found to be 0.004 hr^{-1}.

CONCLUSIONS

The organic pollutants present in the wastewater including pyridine were biologically degraded in a gas–liquid–solid three-phase reactor by utilizing enriched bacterial culture. The culture was developed using waste from a pilot plant used for production of CC2. The bacterium responsible for the degradation of pyridine from the waste mixture was identified as *Bacillus* species. The actual waste was subjected to biodegradation in the slurry bioreactor. It was found to be highly promising and efficient method for the biodegradation of pyridine and other organic pollutants from the waste mixture. The effect airflow rate, Q, and initial biomass concentration (X_0) on the degradation were studied. However, the presence of the residual COD and pyridine after 80 hrs of retention time in the reactor may be due to the non-availability of nutrients, which need further investigation. Eighty two percent degradation of pyridine was achieved within 26 hrs and 88% degradation of pyridine is achieved with in 45 hrs in the reactor. A maximum of 83% COD removal was achieved during the degradation process.

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